

Application of Volcano Plots in Analyses of mRNA Differential Expressions with Microarrays

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ABSTRACT:

In searching differentially expressed mRNAs/genes in a microarray experiment, the two commonly used measures are the fold change and the t -test statistic (or the t -test p -value). The fold-change is a measure of differential expression “signal”, whereas t -statistic is a signal standardized by the noise level, i.e., “signal-to-noise” ratio. The fold-change is an example of absolute effect size, whereas t -test a relative effect size. Both measures have shortcomings: fold change ignores the noise and does not provide an estimation of chance probability; on the other hand, the noise level, thus t -statistic, may not be estimated reliably when the sample size is small. To maximum utilization of statistical information from the data, fold-change and t -statistic can be displayed simultaneously by volcano plots. Volcano plots allow easy comparison between the “double filtering” gene selection criterion and “single filtering” or “joint filtering” criteria. Colored volcano plots provide a flexible way to incorporate external information such as pathway information of a gene. Stratified volcano plots permit examination of hidden patterns such as systematic change of differential expression with the average expression level. Overall, volcano plot is a useful visual tool in microarray analysis.

1 Introduction

The microarray technology allows simultaneous measurements of messenger RNA level of thousands of genes, and its adoption dramatic changes the way biological and biomedical research is carried out (Schena et al., 1998; Young, 2000; Butte, 2002; Slonim, 2002; Stoughton, 2005; Trevino et al., 2007). In particular, the more labor-extensive real-time PCR can be replaced by microarray profiling in a preliminary round, as the general agreement between the two methods is considered to be good (Etienne et al., 2004; Dallas et al., 2005; Morey et al., 2006). As an emerging technology, there are still many issues to be worked out, such as the consistency among different platforms (Park et al., 2004; Larkin et al., 2005; Irizarry et al., 2005; Draghici et al., 2006; Kuo et al., 2006; Patterson et al., 2006; Chen et al., 2007), batch effect (Churchill, 2002; Baggerly et al., 2008; Kitchen et al., 2010), level of noise (Ioannidis, 2005; Ein-Dor et al., 2006), limit of dynamic range (Sharov et al., 2004), etc. However, with better probe design (Yang and Speed, 2002), better data quality control (Shi et al., 2004, 2006), better data reporting requirement (Ioannidis et al., 2009), better normalization scheme (Quackenbush, 2002; Vandesompele et al., 2002; Fujita et al., 2006; Steinhoff and Vingron, 2006; Stafford, 2008; Autio et al., 2009), and better understanding of the study goals, these are not insurmountable problems.

Analyzing large amount of expression data from microarray experiments was thought as a major challenge in early days, but this problem was over-estimated. First, the amount the data from thousands of genes and a hundred or so samples is still much smaller than, e.g., the data generated by whole-genome association studies (Estrada et al., 2009) or next generation sequencing (Schadt et al., 2010), and a moderately sized computer might handle the data without problems. Second, no brand new statistical learning methods had to be re-invented and existing machine techniques could already extract meaningful information from the data (Hastie et al., 2001). Third, the problem of larger number of false positives due to the large number of genes being profiled has been addressed and properly handled (Storey and Tibshirani, 2003; Storey, 2003; Reiner et al., 2003; Pawitan et al., 2005). Fourth, in using multiple genes in constructing classifier, the well known “large p , small n ” problem (large number of variables with small number of sample size) can be solved by the variable/subset/feature/model selection techniques

(Xing et al., 2001; Li and Yang, 2002; Ambroise and McLachlan, 2002; Guyon and Elisseeff, 2003; Li, 2006; Liao and Chin, 2007; Zhao et al., 2010)

One of the most common applications of microarray is “differential expression” profiling: finding mRNAs/genes whose expression level to be very different under two conditions, e.g., with disease and being healthy. Not only could differentially expressed genes provide insight to the biological processes involved in disease etiology, but also these can be used as biomarkers for diagnosis (Golub et al., 1999; Hedenfalk et al., 2001; Dhanasekaran et al., 2001; Adib et al., 2004; Yeatman, 2009) or prognosis (Pomeroy et al., 2002; van de Vijver et al., 2002; Colman et al., 2010; Kim and Paik, 2010). The phrase “differential expression” means that the *averaged* expression level of a mRNA/gene in one phenotype-specific group is much *larger* or *smaller* than that in another group. However, the terms “average” and “larger/smaller” are up to various interpretations.

There are at least two definitions of average: arithmetic mean or geometric mean. For a random variable x , arithmetic mean can be represented by $E[x]$, $\langle x \rangle$, or \bar{x} , which is equal to $\frac{1}{n} \sum_{i=1}^n x_i$ (where n is the sample size). Geometric mean is defined by $(x_1 x_2 \cdots x_n)^{1/n}$. For fluorescence-light-intensity based microarray data x , it is a common practice to logarithmically transform the data $x' = \log(x)$, because x' fits better than x to a normal distribution. Then arithmetic mean of x' is actually equal to the logarithm of geometric mean of x : $E[x'] = \frac{1}{n} \sum_{i=1}^n \log(x_i) = \log(x_1 x_2 \cdots x_n)^{1/n}$.

Deciding “how larger one group’s average is compared to the other” is no less trivial. Fold change and t -statistic are the two main choices for measure of differential expression. In microarray analysis field, these two measures have been in and out of favor at various time. Fold change had been commonly used before it was pointed out that it did not take the noise into account (Chen et al., 1997; Baldi and Long, 2001). t -statistic enjoyed its acceptance until another round of papers suggesting that genes selected by fold-change are more consistent among different microarray platforms than those selected by t -statistics (Shi et al., 2005, 2006; Guo et al., 2006). This result triggered more comments on the relationship between reproducibility and accuracy, and between biological and statistical signal (Witten and Tibshirani, 2007).

If we recognize that both fold change and t -statistic have advantages and shortcomings,

then both pieces of information should be used in an analysis. The problem with fold change is that the same fold change value will be less impressive if the variance is large. Although t -statistic aims at taking the noise level into account, the practical problem is that the variance may not be estimated reliably, especially when the sample size is small. Volcano plot, the topic of this review, is a visual tool to display both fold change and t -statistic.

This article is organized as follows: Section 2 establishes a relationship between the fold-change and t -statistic; Section 3 introduces volcano plots which simultaneously display both fold-change and t -statistic; Section 4 introduces the modified t -statistic which tends to reduce the gene-to-gene variation of variance estimation; and Section 5 is the discussion section. One microarray dataset is used throughout this paper, which consists of 37 chronic lymphocytic leukemia (CLL) samples and 17 control samples. The expression profiling has been carried out on Illumina platform with 48804 probesets.

2 Fold change and t -statistic: signal and signal-to-noise ratio

Fold change (FC) and t -statistic seem to be two very different quantities: one is intuitive and a straightforward measure of differences, another is rooted deeply in field of statistics. However, with logarithm transformation there is a relationship between the two.

The need for logarithmic transformation can be illustrated by Fig.1. Fig.1 shows the three histograms of fluorescence-light intensity E of a microarray experiment which is indicative of the number of mRNA copies hybridized to the probe, thus a measure of mRNA expression level: (A) in regular scale, (B) in log-transformed x -axis scale, and (C) of $\log(E)$ itself. Without the logarithmic transformation, the distribution of E is very long-tailed, and very skewed (asymmetric). With the log transformation (or other similar transformations), even though the distribution is still not a perfect normal distribution, it is much more normal-like.

There are other advantages of a log transformation, e.g. variance is more stablized and does not tend to increase with the mean; it is consistent with a psycho-physics law relating human sensation to the logarithm of the stimulus level (Fechner, 1860). Note that in a future technology where the number of copies of mRNA can be read directly without fluorescence light intensity as the intermediate (Geiss et al., 2008; Robinson et al., 2010), the role of log

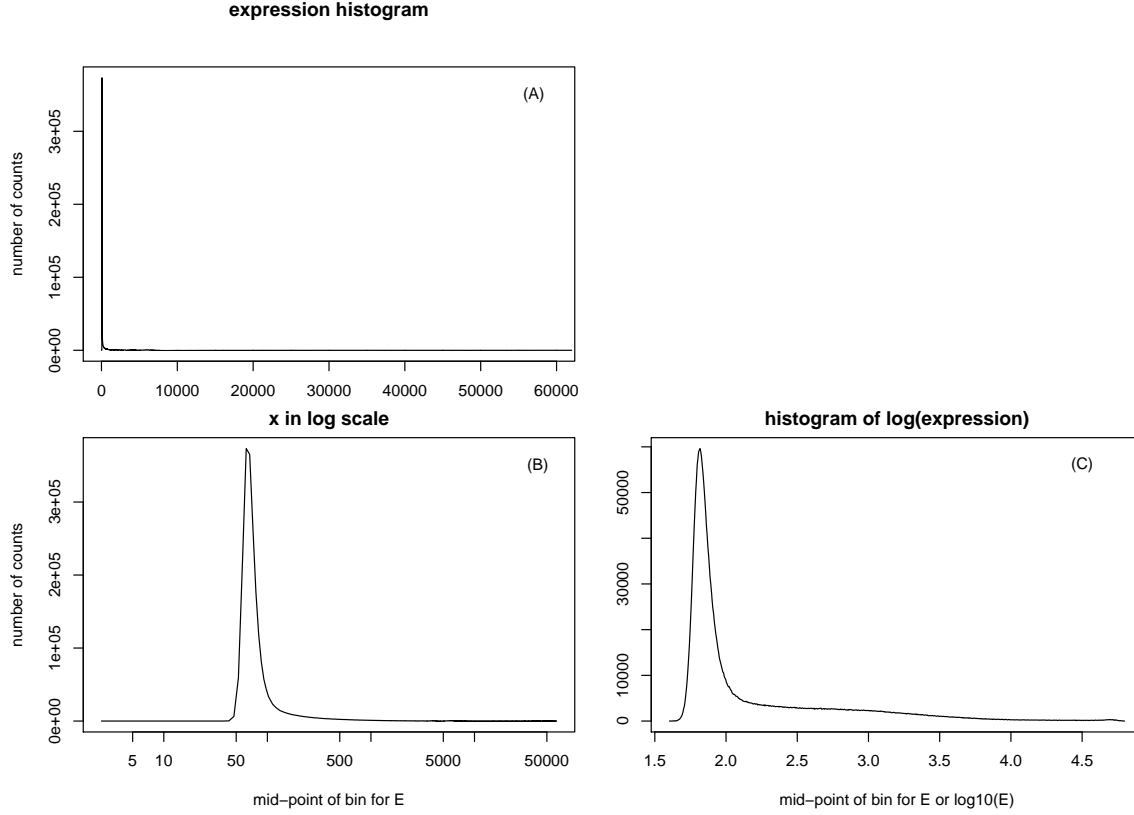


Figure 1: Histogram of expression levels of a microarray experiment (this unpublished dataset contains 37 case samples, 18 control samples, and 48804 probesets in Illumina platform, normalized by “quantile normalization”): (A) in linear scale. (B) x -axis in a log scale. (C) for log-transformed expression.

transformation might be reconsidered. However, the decision on whether to log transform or not is still based on the histogram of E vs. that of $\log(E)$.

The simplest definition of FC is:

$$FC = \frac{\langle E_1 \rangle}{\langle E_0 \rangle}, \quad (1)$$

where the arithmetic average is over the fluorescence-light intensity of samples in group 1 (e.g. diseased group) and group 0 (e.g. control group). The logarithm of FC is:

$$\log(FC) = \log \frac{\langle E_1 \rangle}{\langle E_0 \rangle} = \log \langle E_1 \rangle - \log \langle E_0 \rangle \approx \langle \log E_1 \rangle - \langle \log E_0 \rangle. \quad (2)$$

Switching the order of averaging and log-transformation usually does not lead to identical values, so the above expression is only approximately true. We can have a second definition of

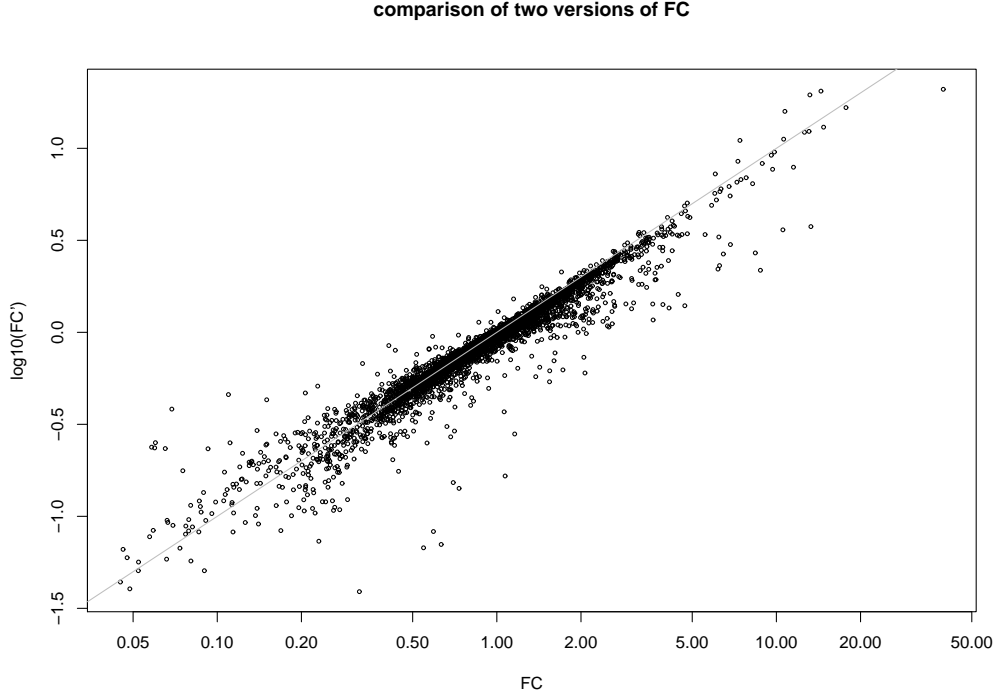


Figure 2: Comparison of two definitions of fold changes. The x is FC defined in Eq.(1), in log scale. The y is the $\log(FC')$ defined in Eq.(3).

FC called FC':

$$\log(FC') = \langle \log E_1 \rangle - \langle \log E_0 \rangle \quad (3)$$

Fig.2 shows that FC is mostly similar to FC' and we do not distinguish the two definitions. The same conclusion is also reached in (Witten and Tibshirani, 2007).

The t -test is an example of statistical testing whose goal is to compare any observed result with chance events. The statistic used in t -test (e.g. (Snedecor and Cochran, 1989)) is the difference of arithmetic means in two groups divided ("standardized") by the estimated standard deviation of that difference. Standard deviation of parameters (e.g., sample mean, sample variance) is often called "standard error" (SE) (Snedecor and Cochran, 1989). One requirement for using t -test is that values in two groups roughly follow normal distributions (with different means). As discussed above, we need to log transform the fluorescence light intensity E to have a normal-like distribution, so t -statistic is:

$$t = \frac{\langle \log E_1 \rangle - \langle \log E_0 \rangle}{SE_{\langle \log E_1 \rangle - \langle \log E_0 \rangle}}. \quad (4)$$

The commonly used estimation of SE , due to Welsh (Welsh, 1947), assumes different variances in group 1 and group 0:

$$t_{welsh} = \frac{\langle \log E_1 \rangle - \langle \log E_0 \rangle}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_0^2}{n_0}}} \quad (5)$$

where s_1^2 and s_0^2 are the estimated variances (of $\log(E)$) of group 1 and 0, and n_1, n_0 are number of samples in the two groups. For readers who are not familiar with statistics, the following points can be used to understand the pooled estimation of SE in the denominator of Eq.5:

(1) SE is the square root of variance and variance is the square of SE ; (2) variance of sum or difference of two variables, $Var[x_1 \pm x_2]$, is the sum of individual variances $Var[x_1] + Var[x_2]$; (3) SE of sample means is sample standard deviation divided by \sqrt{n} (Snedecor and Cochran, 1989).

The last point can be particularly hard to grasp for biologists, but we are dealing with two different types of mean and standard deviations here. For a dataset with n samples, the mean, standard deviation, variance are $E[x]$ (or $\langle x \rangle, \mu$), $Sd[x]$ (or $\sqrt{Var[x]}, s$), $Var[x]$ (or s^2). When the dataset is hypothetically replicated many times, we can talk about mean, standard deviation, variance of the sample-mean μ , as each replicate of the dataset may not be exactly the same. The dataset-mean is the same as sample-mean, but the dataset-standard-error is s/\sqrt{n} and dataset-variance is s^2/n (Snedecor and Cochran, 1989).

This section establishes a relationship between $\log(FC)$ and t -statistic: t is $\log(FC)$ (or more accurately, $\log(FC')$) standardized by the noise level as measured by the pooled standard error. In the field of statistical behavioral science, quantitative psychology, epidemiology, and meta-analysis, there is a similar theme of unstandardized vs. standardized effect size (Cohen, 1988). In the field of engineering, quantities like t can be called a signal-to-noise ratio (another definition of signal-to-noise ratio is based on power ratio, thus a square operation is applied). In the field of applied probability, mean divided by standard deviation is the inverse of coefficient of variation.

3 Volcano plot and its basic use

If the noise level is known or can be reliably estimated, we of course prefer the measure

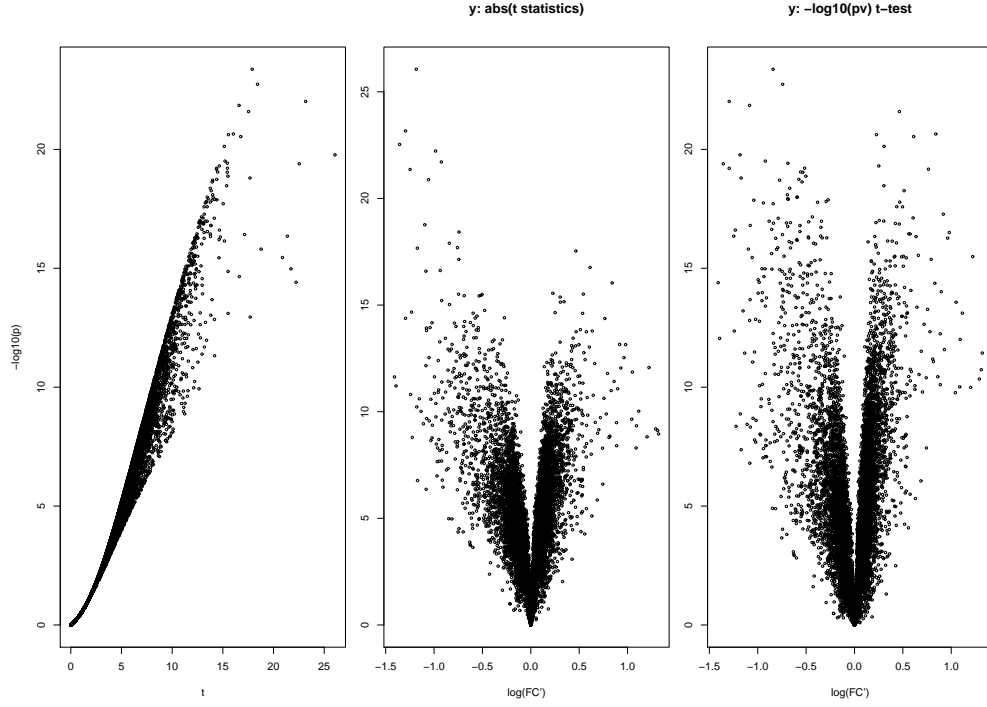


Figure 3: (A) x -axis: t -statistic, y -axis: $-\log_{10}(p\text{-value})$ of t -test. (B) Volcano plot using t -statistic in y -axis (x -axis is $\log_{10}FC$). (C) Volcano plot using $-\log_{10}(p\text{-value})$ in y -axis.

of differential expression that takes the noise level into account, such as t -statistic. In reality, not only is smaller sample sizes an issue for variance estimation, but also, if systematic error exists, we may not improve the situation by increasing the sample size. For example, it is observed that noise level during the hybridization stage is much higher than that during the sample preparation or amplification stage (Tu et al., 2002). If a probe sequence for an mRNA is highly represented in the genome, cross-hybridization can be a cause of error and variation. However, the probability of this error does not seem to decrease with large sample sizes.

Facing this reality, we might just display and use both FC and t -statistic, and this is the volcano plot. Volcano plot most often refers to the scatter-plot with $-\log_{10}(p\text{-value})$ from the t -test as the y -axis and $(\log_{10})FC$ as the x -axis (Jin et al., 2001; Cui and Churchill, 2003). However, t -statistic and $-\log_{10}(p\text{-value})$ is (see Fig.3(A)) is highly correlated, and whether the t (Fig.3(B)) or $-\log_{10}(p\text{-value})$ (Fig.3(C)) is used in the y -axis, the outcome is very similar. The reason why t and p -value from t -test is not one-to-one corresponding (Fig.3(A)) is because in determining p -value, Welch's t distribution has a degree of freedom parameter which also

depends on the data (Pan, 2002).

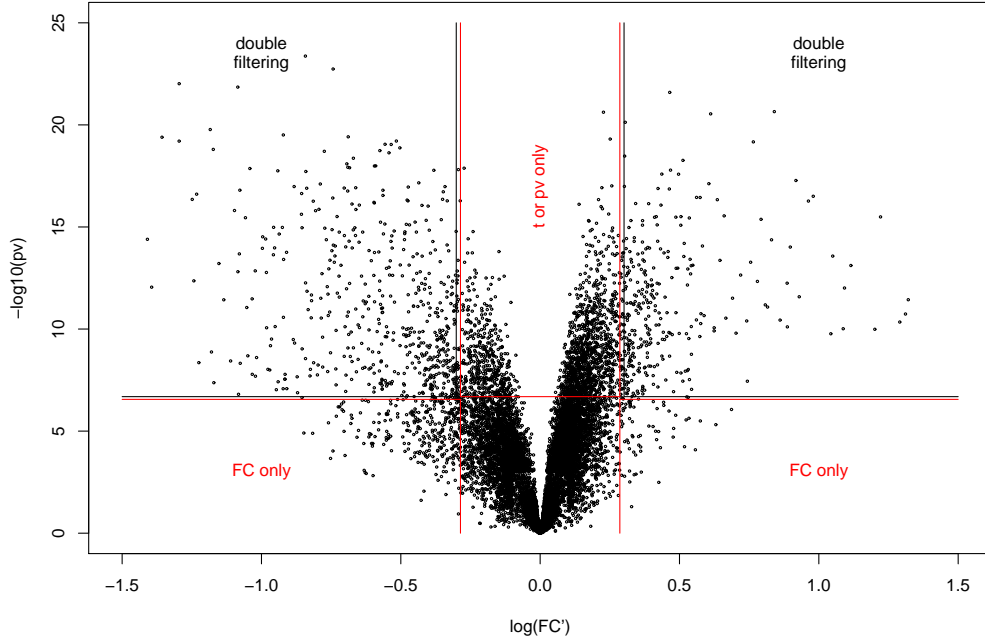


Figure 4: Illustration of the double filtering criterion (upper-left and upper-right corners delineated by black lines), FC-only single-gene criterion (lower-left and lower-right corners delineated by red lines), and t -test-only single-gene criterion (“football goalpost” in the middle delineated by red lines).

The basic use of volcano plots is to check genes that could be selected by one differential expression criterion but not the other. The familiar “double filtering” (Zhang and Cao, 2009) used by many groups is to set the gene selection criterion by: (i) $|\log_{10} FC| > \log_{10} FC_0$; and (ii) $t > t_0$. Equivalently, it can be defined as (i) $|\log_{10} FC| > \log_{10} FC_0$; and (ii) $p\text{-value} < p_0$. FC_0 , t_0 , p_0 are preset threshold values for fold change, t -statistic, and t -test p -value. The double filtering criterion corresponds to a cutting of two outer rectangular regions in the volcano plot (Fig.4).

The single filtering criterion, after removing the double criterion selected genes, corresponds to rectangular regions along the two axes (Fig.4). These are often the genes not selected for reasonable arguments: (i) genes with large fold change but nevertheless insignificant test result may be caused a few outliers with very large values in one group. (ii) genes with good test result (large t ’s and small t -test p -values) but low fold change could be false signal due to low variance, which can be caused by batch effect (Leek et al., 2010), or low expression level (to

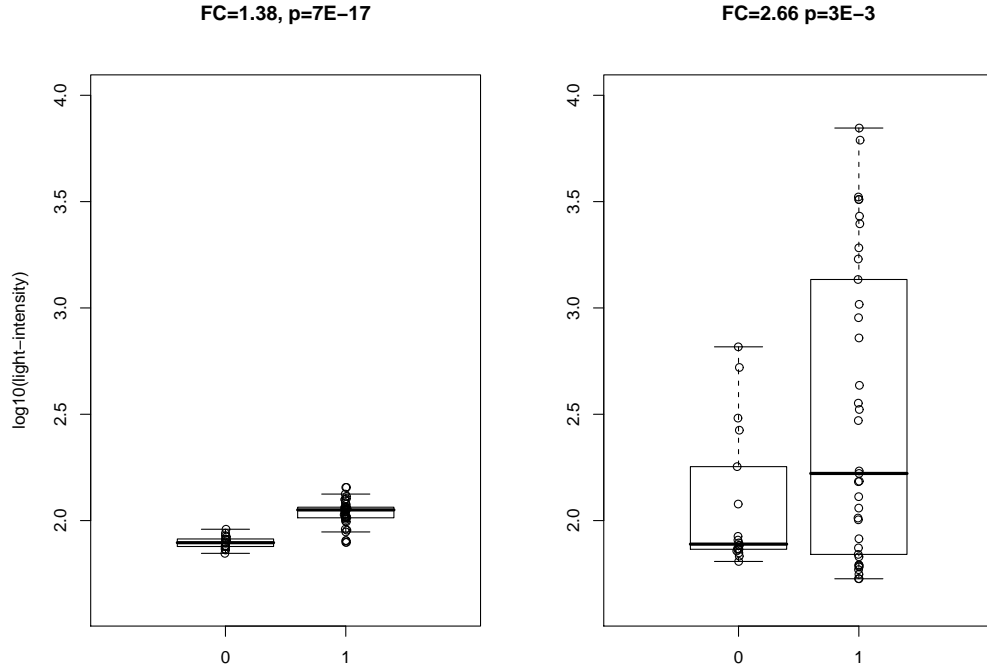


Figure 5: (A) a gene with very good t -test result (p -value = 7.7×10^{-17}) but only moderate fold-change (FC=1.38). (B) a gene with large fold-change (FC=2.66) but moderate t -test result (p -value= 3×10^{-3}).

be discussed later).

The goal of using double filtering criterion is to obtain a more robust result. The cost we pay is that some real differentially expressed genes might be missed. Volcano plot allows us to pick some genes from the single filtering region for further examination. Fig.5 shows two examples of genes selected by single filtering criterion.

Fig.5(A) is gene selected by t -test p -value only ($p = 7.7 \times 10^{-17}$) while FC is lower than 2 (FC=1.379). If the true variance is indeed low and we estimated it correctly from 17 control samples, then we trust that this gene is significantly differentially expressed. Fig.5(B) is selected by FC only (FC=2.66) whereas the p -value is only 3×10^{-3} . This gene can still be a significantly differential-expression if the large variance in the case group is due to something else, e.g. sub-disease types. Statistical analysis alone should not be the only foundation for selecting potentially relevant genes, and volcano plot is a way to pick those genes which otherwise might be missed.

4 Robust variance estimation, regularization, SAM, and joint filtering

The essential difference between FC and t -statistic is the consideration of statistical noise (variance), but the real challenge is how to estimate the variance from a small number of samples. Since variance is calculated around the mean which is also estimated, one idea for robust variance estimation is to iteratively remove outliers then calculate mean and variance (Dozmorov and Lefkovits, 2009). The drawback of this approach is that the number of samples used is gradually reduced.

Another idea for robust variance estimation is motivated by the typical “large p small n ” situation for a microarray experiment (Li and Yang, 2002). Though the sample size n could be small, the number of genes p is nevertheless large, and that large number of genes make it possible for a reliable estimation of common variance cross all genes (Pan, 2002; Cui et al., 2005), at least for the control group.

One main worry about variance estimation is that its value can be low due to the low expression level. To avoid the estimated variance being too low, we may add a constant “penalty” term s_0 to the sample-estimated standard deviation (Tusher et al., 2001):

$$t_{sam} = \frac{\langle \log E_1 \rangle - \langle \log E_0 \rangle}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_0^2}{n_0} + s_0}}. \quad (6)$$

The penalty is also called “regularization”, reflecting the prior belief (in the Bayesian framework) that variance estimation across different genes should exhibit certain smooth behavior (Baldi and Long, 2001; Hastie et al., 2001).

A popular software package called SAM (Statistical Analysis of Microarray) (<http://www-stat.stanford.edu/>) is based on Eq.(6). Another R (R is a free software environment for statistical computing: <http://www.r-project.org/>) implementation of the same idea, *siggen*, can be found at <http://www.bioconductor.org/packages/2.3/bioc/html/siggenes.html>. In SAM, detailed procedures are proposed to determine the s_0 value from the data. It is not clear whether this procedure is unique or it is just one of many options. In practice, any small value of s_0 , such as the 5% percentile of standard deviations of all genes, can stabilize the variance estimation.

A Bayesian derivation of the extra term in variance estimation is derived in (Baldi and Long,

2001). In this framework, mean, variance of a normal distribution (of $\log(x) = x'$) has a prior distribution, as well as a posterior distribution after data are observed. For convenience, they pick a functional form of prior distribution so that posterior will have the same functional form (inverse Gamma distribution for the variance, normal distribution for the mean). It can be shown that (the mean of) posterior variance is a weighted sum of prior variance (σ_0^2) and the sample-estimated of variance s^2 (Baldi and Long, 2001):

$$\sigma_{mean.of.posterior}^2 = ws^2 + (1 - w)\sigma_0^2 \quad (7)$$

where weight w tend to close to 1 for larger sample size ($w = (n - 1)/(\nu_0 + n - 2)$, ν_0 is the prior degree of freedom for the inverse Gammar distribution).

The modified or regularized variance $\sigma_{mean.of.posterior}^2$ in Eq.(7) has the effect of drawing gene-specific variance towards the middle, since the change from the estimated variance:

$$\sigma_{mean.of.posterior}^2 - s^2 = ws^2 + (1 - w)\sigma_0^2 - s^2 = -(1 - w)(s^2 - \sigma_0^2), \quad (8)$$

is negative when $s^2 > \sigma_0^2$ and positive when $s^2 < \sigma_0^2$. Note that variance is added in Eq.(7), as versus standard deviations being added in the denominator in Eq.(6). However, the idea of adding an extra constant term is the same in Eq.(6) and Eq.(7).

In fact, there is a second extra term in variance estimation if the sample-estimated mean is not a good estimate of the true mean (Baldi and Long, 2001). For this reason, it is reasonable to consider removing outliers to make sure the mean is estimated robustly (Dozmorov and Lefkovits, 2009).

What is the relationship between robust variance estimation and volcano plots? FC can be considered to be the special case when variances of all genes are equal, t -statistic of course contains gene-specific variance, and t_{sam} in Eq.(6) is somewhere in-between. Rewrite $|\langle \log E_1 \rangle - \langle \log E_0 \rangle|$ as δ , $\sqrt{s_1^2/n_1 + s_0^2/n_0}$ as s , the regularized t -statistic in Eq.(6) can be split into two terms (Zhang and Cao, 2009):

$$t_{sam} = \frac{\delta}{s + s_0} = \frac{1}{2(s + s_0)}\delta + \frac{s}{2(s + s_0)}\frac{\delta}{s} = \frac{1}{2(s + s_0)}|\log(FC')| + \frac{s}{2(s + s_0)}t_{welsh}. \quad (9)$$

In other words, t_{sam} is a weighted sum of $\log(FC')$ and t -statistic.

A constant t_{sam} value corresponds a line in the volcano plot: $w|x| + vy = t_{0,sam}$, where $w = 0.5/(s + s_0)$, $v = 0.5s/(s + s_0)$. Gene selection criterion by SAM (Eq.(6) and Eq.(9)) is

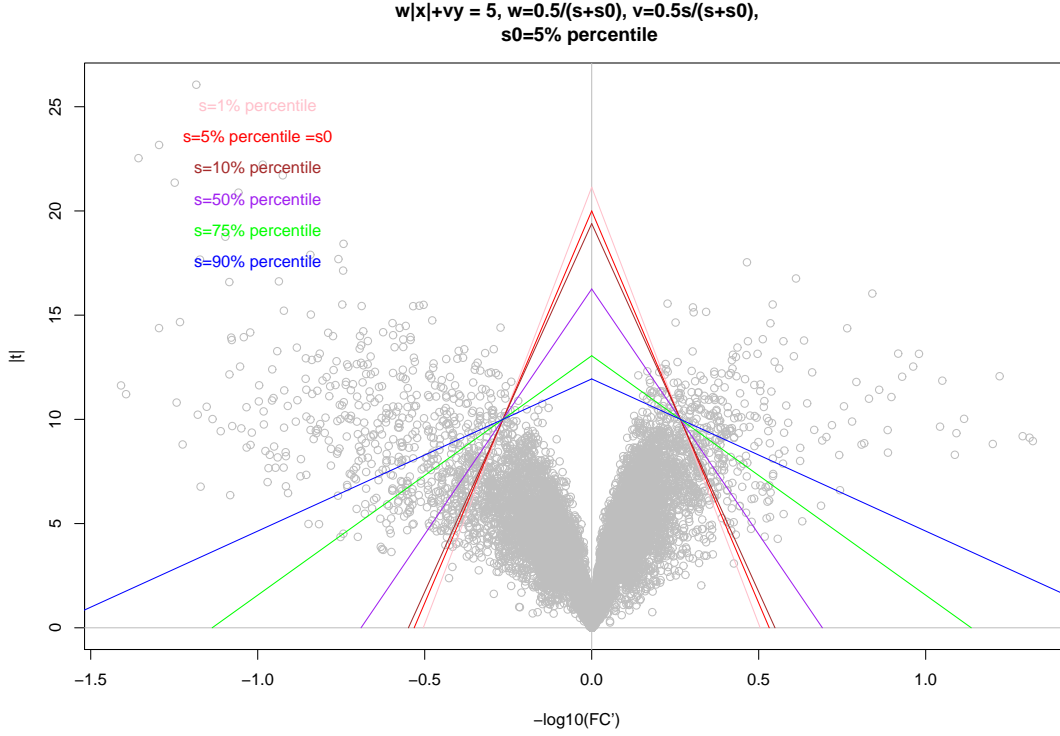


Figure 6: All lines correspond to the constant $t_{sam} = 5$ value (Eq.(6)) with $s_0 = 0.0266$ being the 5% percentile standard deviation of all 48804 probes/genes. The six lines are for genes with different standard deviations: $s = 0.0238, 0.0266, 0.0283, 0.0425, 0.0871, 0.137$ (1%, 5%, 10%, 50%, 75%, 90% percentiles, pink, red, brown, purple, green and blue).

$t_{sam} > t_{0,sam}$. Because each gene has its own standard deviation value s , the threshold can be gene-specific. We illustrate this important property of SAM in Fig.6. The s_0 is set at 0.0266 which is the 5% percentile value of s 's of all genes in our dataset. For a gene with standard deviation of $s = 0.0238, 0.0266, 0.0283, 0.0425, 0.0871, 0.137$ (1%, 5%, 10%, 50%, 75%, 90% percentiles), the $t_{sam} = 5$ threshold is represented by lines with various slopes (pink, red, brown, purple, green, blue in Fig.6).

The lines for low-variance genes have steeper slopes, indicating that FC plays a more important role in differential expression gene selection. On the other hand, for high-variance genes, the threshold lines have flatter slope, indicating that t -test result is more important. As discussed in the previous section (and Fig.5), low-variance genes tend to have low FC values and high-variance genes tend to have less significant test result, so the consequence of using SAM is to counter-balance this trend and to obtain a more robust outcome. We also note that

the SAM-based gene selection regions in Fig.6 (complementary to triangles) are very different from those by double-filtering criterion (rectangles in Fig.5). This can also be called a “joint filtering” criterion (Zhang and Cao, 2009).

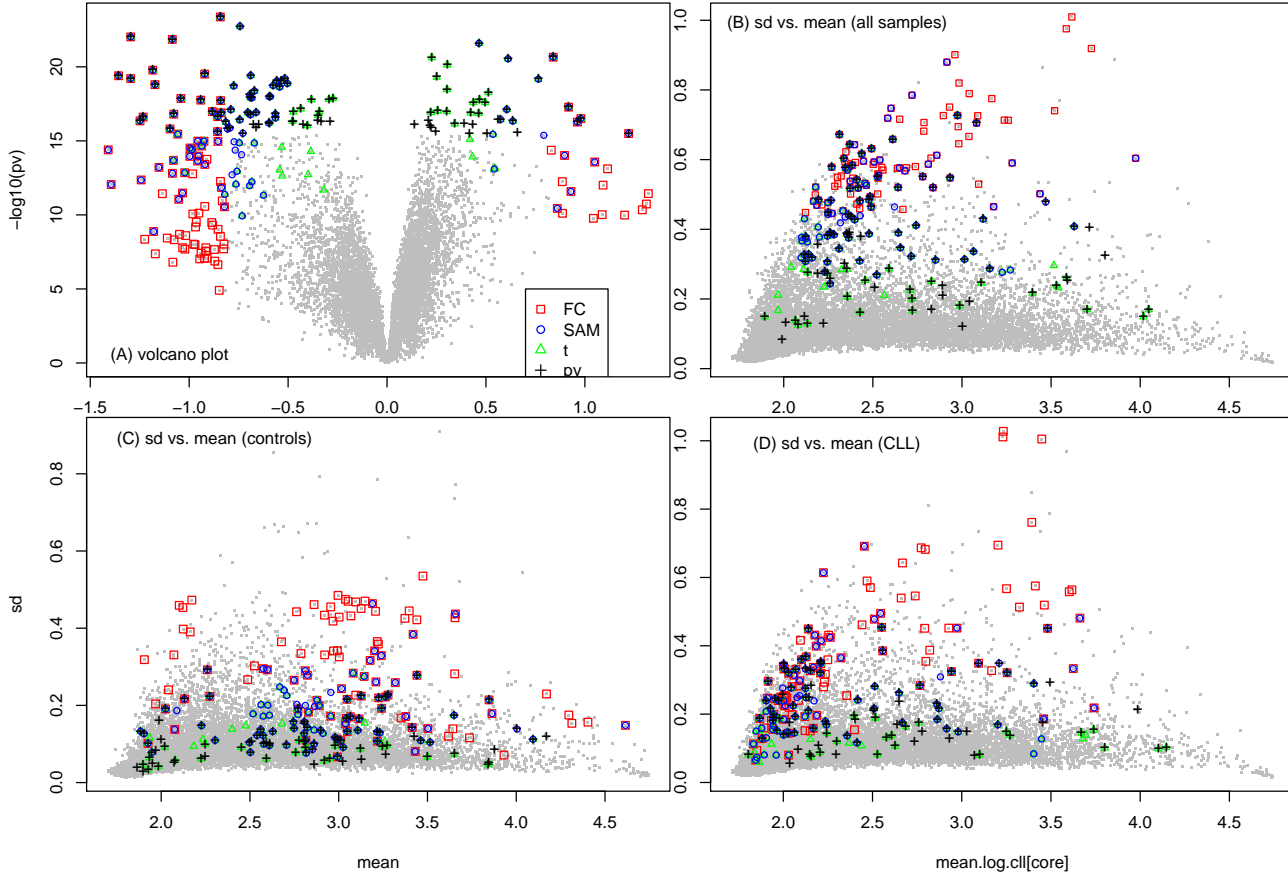


Figure 7: Green, red, green, black dots are the top 100 probes/genes selected by t_{sam} , FC' , t -statistic, and p -value of t -test. (A) on volcano plot, x : $\log(FC')$, y : $-\log_{10}(p\text{-value})$. (B) x : mean of all samples, y : standard deviation of all samples. (C) x : mean of control samples, y : standard deviation of control samples. (D) x : mean of diseased samples, y : standard deviation of diseased samples.

Fig.7(A) compares the top 100 genes selected by SAM (regularized t) (blue) with those selected by FC (red), t -test p -value (black), and t -statistic itself (green). Although there are certain overlaps among different selection criteria, SAM is able to pick up genes that are not selected by either FC or t -test p -value alone.

To address another question on whether t -test criterion tends to select genes with low

variance and low expression level. Fig.7(B)(C)(D) show the standard deviation (y -axis) vs. mean (x -axis) for all samples, control samples only, and diseased (CLL) samples only. Indeed, FC-based criterion tend to select genes with high variances, whereas t -test based criterion selects relatively low variance genes. SAM achieves a balance between the two criteria, and selects genes with intermediate variance values. On the other hand, there is no strong evidence that any selection criterion tends to select low expression level genes.

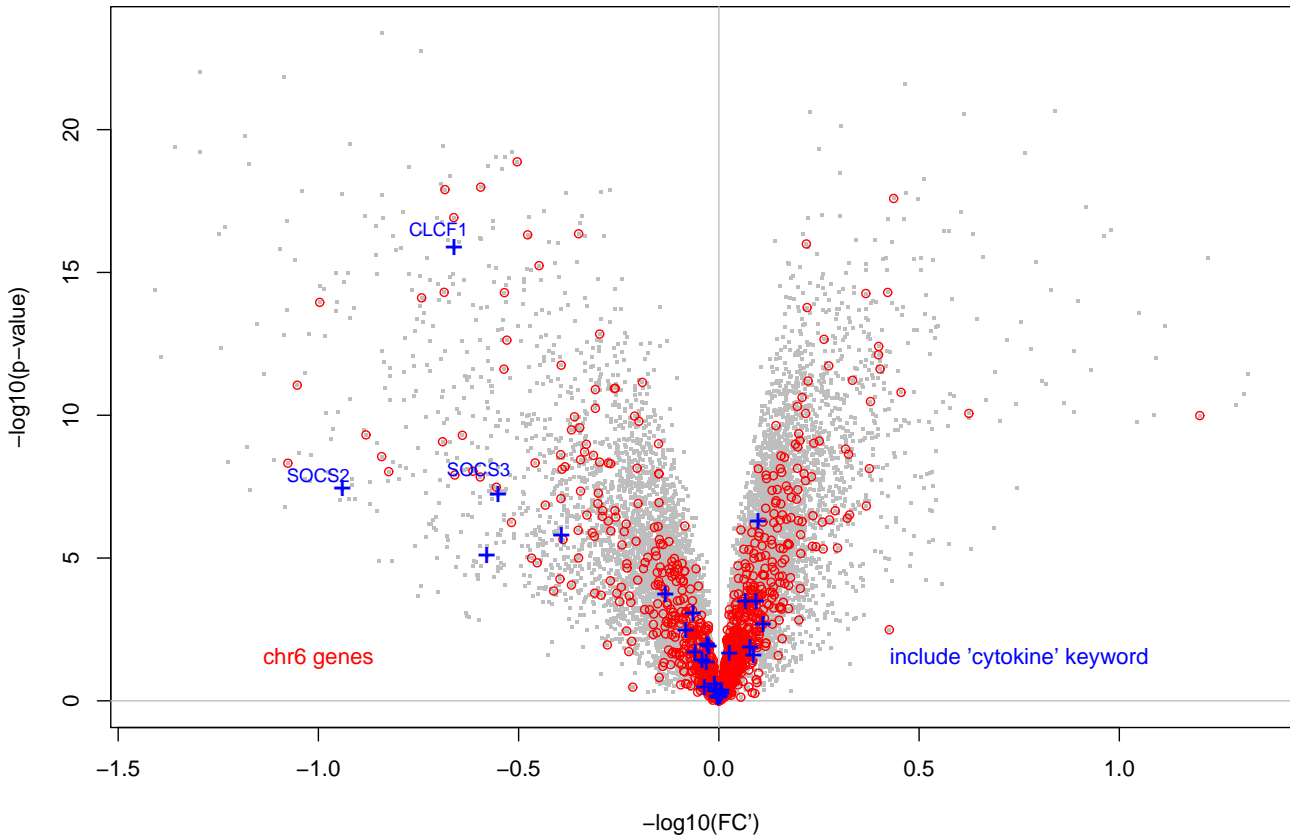


Figure 8: Stratified volcano plot: probes/genes on chromosome 6 are marked by red, and those with “cytokine” in gene annotation is marked by blue.

5 Discussion

The idea and the use of volcano plots can be expanded in several directions. First of all, as a 2-dimensional plot, with potentially interesting genes scattered outward, one can examine

any external information by introducing colors. If that external piece of information is relevant to differential expression, we can easily recognize the fact by a visual impression of the plot. This coloring of a volcano plot can be called “stratified volcano plot”. One example is to label all probes/genes that belong to a particular pathway, cellular component, function, or process coded in GO (gene ontology) categories (Ashburner et al., 2000).

Fig.8 illustrates a stratified volcano plot by marking 1614 probes/genes that are located on chromosome 6 (red), and 31 probes/genes whose annotation contains the word “cytokine”. From the stratified volcano plot, we can easily identify interesting candidate genes involving cytokines such as CLCF1 (cardiotrophin-like cytokine factor 1, p -value= 1.4×10^{-16} , $FC'=0.22$, down-regulated), SOCS2 (suppressor of cytokine signaling 2, $FC'=0.11$, p -value= 3.8×10^{-8} , down-regulated), SOCS3 (suppressor of cytokine signaling 3, $FC'=0.28$, p -value= 6.2×10^{-8} , down-regulated), etc.

In a work-in-progress, we have developed an *R* package to color a volcano plots using the average expression levels (Hua et al., 2011). In the program, we introduced an interactive feature for users to click a probe/gene on the volcano plot to show the gene names or other information.

Secondly, the idea of simultaneously display of noise-level-standardized signal and unstandardized one can be useful beyond the microarray field. In genetic association studies, the association signal of a single-nucleotide polymorphism (SNP) is usually measured by two quantities. One is the odds-ratio (OR) of the 2-by-2 count table with disease status as row and two alleles as column. OR is not standardized by the noise level or sample size, though the 95% confidence interval of OR does become narrower for larger sample sizes thus lower level of chance events (Li, 2006). On the other hand, the chi-square statistic or the p -value of the chi-square (χ^2) test strongly dependent on sample size, thus chance event probability. In fact, the chi-square statistics is proportional to the total number of samples for a SNP that contains association signal.

Besides using OR in x -axis (in log scale), another choice is to use the allele frequency difference in case and control group. Denote the four counts in the 2-by-2 table in case-control association analysis are a, b, c, d , $\log_{10}OR$ is $\log_{10}(ad) - \log_{10}(bc)$, whereas allele frequency difference is $a/(a+b) - c/(c+d) = (ad-bc)(a+b)^{-1}(c+d)^{-1}$. In other words, the difference

between the two choices is whether ad and bc are compared in the logarithmic or regular scale.

It is rare to find a genetic association paper that applies volcano plots (Sirota et al., 2009; Miclaus et al., 2010). We believe that many extensions and applications of volcano plots in microarray analysis can be equally useful in genetic association analysis. For example, the joint filtering criterion, the stratified volcano plot coloring external pieces of information, and uncovering of systematic patterns when the colorings are on other statistical information. We have found that the location of a SNP on the volcano plot is intrinsically related to its minor allele frequency. This will provide further insight on how one should balance the chi-square test result and odds-ratio in selecting genetically associated genes.

In conclusion, volcano plot displays both noise-level-standardized and unstandardized signal concerning differential expression of mRNA levels. Joint filtering has a simple geometric interpretation in volcano plot, and its advantage over double filter criterion of genes can be easily understood. As a scattering plot, volcano plot can incorporate other external information, such as gene annotation, to aid the hypothesis generating process concerning a disease.

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References

- TR Adib, S Henderson, C Perrett, D Hewitt, D Bourmpoulia, J Ledermann, C Boshoff (2004), “Predicting biomarkers for ovarian cancer using gene-expression microarrays”, *British Journal of Cancer*, 90:686-692.
- C Ambrose, GJ McLachlan (2002), “Selection bias in gene extraction on the basis of microarray gene-expression data”, *Proceedings of National Academy of Sciences*, 99:6562-6566.

- M Ashburner, CA Ball, JA Blake, D Botstein, H Butler, JM Cherry, AP Davis, K Dolinski, SS Dwight, JT Eppig, MA Harris, DP Hill, L Issel-Tarver, A Kasarskis, S Lewis, JC Matese, JE Richardson, M Ringwald, GM Rubin, G Sherlock (2000), “Gene Ontology: tool for the unification of biology” *Nature Genetics*, 25:25-29.
- R Autio, S Kilpinen, M Saarela, O Kallioniemi, S Hautaniemi, J Astola (2009), “Comparison of Affymetrix data normalization methods using 6,926 experiments across five array generations”, *BMC Bioinformatics*, 10(suppl 1):S24.
- KA Baggerly, KR Coombes, ES Neeley (2008), “Run batch effects potentially compromise the usefulness of genomic signatures for ovarian cancer” *Journal of Clinical Oncology*, 26:1186-1187.
- P Baldi, AD Long (2001), “A Bayesian framework for the analysis of microarray expression data: regularized t -test and statistical inferences of gene changes”, *Bioinformatics*, 17:509-519.
- A Butte (2002), “The use and analysis of microarray data”, *Nature Reviews Drug Discovery*, 1:951-960.
- Y Chen, ER Dougherty, ML Bittner (1997), “Ratio-based decisions and the quantitative analysis of cDNA microarray images” *Journal of Biomedical Optics*, 2:364-274.
- JJ Chen, HM Hsueh, RR Delongchamp, CJ Lin, CA Tsai (2007), “Reproducibility of microarray data: a further analysis of microarray quality control (MAQC) data”, *BMC Bioinformatics*, 8:412.
- G Chu, B Narasimhan, R Tibshirani, V Tusher (2007), *SAM: “significance analysis of microarrays”, users guide and technical document*, v.3.0.
- GA Churchill (2002), “Fundamentals of experimental design for cDNA microarrays”, *Nature Genetics*, 32:490-495.
- J Cohen (1988), *Statistical Power Analysis for the Behavioral Sciences*, 2nd edition (Lawrence Erlbaum Associates, Inc.).

- H Colman, L Zhang, EP Sulman, JM McDonald, NL Shooshtari, A Rivera, S Popoff, CL Nutt, DN Louis, JG Cairncross, MR Gilbert, HS Phillips, MP Mehta, A Chakravarti, CE Pelloso, K Bhat, BG Feuerstein, RB Jenkins, K Aldape (2010), “A multigene predictor of outcome in glioblastoma”, *Neuro-Oncology*, 12:49-57.
- X Cui, GA Churchill (2003), “Statistical tests for differential expression in cDNA microarray experiments”, *Genome Biology*, 4:210.
- X Cui, JTG Hwang, J Qiu, NJ Blades, GA Churchill (2005), “Improved statistical tests for differential gene expression by shrinking variance components estimates”, *Biostatistics*, 6:59-75.
- PB Dallas, NG Gottardo, MJ Firth, AH Beesley, K Hoffmann, PA Terry, JR Freitas, JM Boag, AJ Cummings, UR Kees (2005), “Gene expression levels assessed by oligonucleotide microarray analysis and quantitative real-time RT-PCR – how well do they correlate?”, *BMC Genomics*, 6:59.
- SM Dhanasekaran, TR Barrette, D Ghosh, R Shah, S Varambally, K Kurachi, KJ Pienta, MA Rubin, AM Chinnaiyan (2001), “Delineation of prognostic biomarkers in prostate cancer”, *Nature*, 412:822-826.
- I Dozmorov, I Lefkovits (2009), “Internal standard-based analysis of microarray data. Part 1: analysis of differential gene expressions”, *Nucleic Acids Research*, 37:6323-6339.
- S Draghici, P Khatry, AC Eklund, Z Szallasi (2006), “Reliability and reproducibility issues in DNA microarray measurements”, *Trends in Genetics*, 22:101-109.
- L Ein-Dor, O Zuk, E Domany (2006), “Thousands of samples are needed to generate a robust gene list for predicting outcome in cancer”, *Proceedings of National Academy of Sciences*, 103:5923-5928.
- K Estrada, A Abuseiris, FG Grosveld, AG Uitterlindern, TA Knoch, F Rivadeneira (2009), “GRIMP: a web- and grid-based tool for high-speed analysis of large-scale genome-wide association using imputed data”, *Bioinformatics*, 25:2750-2752.

- W Etienne, MH Meyer, J Peppers, RA Meyer Jr. (2004), “Comparison of mRNA gene expression by RT-PCR and DNA microarray” *BioTechniques*, 36:618-626.
- GT Fechner (1860), *Elemente der Psychophysik* (Leipzig: Breitkopf und Härtel).
- A Fujita, JR Sato, L de Oliveira Rodrigues, CE Ferreira, MC Sogayar (2006), “Evaluating different methods of microarray data normalization”, *BMC Bioinformatics*, 7:469.
- GK Geiss, (2008), “Direct multiplexed measurement of gene expression with color-coded probe pairs”, *Nature Biotechnology*, 26:317-325.
- TR Golub, DK Slonim, P Tamayo, C Huard, M Gaasenbeek, JP Mesirov, H Coller, ML Loh, JR Downing, MA Caligiuri, CD Bloomfield, ES Lander (1999), “Molecular classification of cancer: class discovery and class prediction by gene expression monitoring”, *Science*, 286:531-537.
- L Guo, EK Lobenhofer, C Wang, R Shippy, SC Harris, L Zhang, N Mei, T Chen, D Herman, FM Goodsaid, P Hurban, KL Phillips, J Xu, X Deng, YA Sun, W Tong, YP Dragan, L Shi (2006), “Rat toxicogenomic study reveals analytical consistency across microarray platforms”, *Nature Biotechnology*, 24:1162-1169.
- I Guyon and A Elisseeff (2003), “An introduction to variable and feature selection”, *Journal of Machine Learning Research*, 3:1157-1182.
- T Hastie, R Tibshirani, J Friedman (2001), *The Elements of Statistical Learning* (Springer).
- I Hedenfalk, D Duggan, Y Chen, M Radmacher, M Bittner, R Simon, P Meltzer, B Gusterson, M Esteller, M Raffeld, Z Yakhini, A Ben-Dor, E Dougherty, J Kononen, L Bubendorf, W Fehrle, S Pittaluga, S Gruvberger, N Loman, O Johannsson, H Olsson, B Wilfond, G Sauter, OP Kallioniemi, A Borg, J Trent (2001), “Gene-expression profiles in hereditary breast cancer”, *New England Journal of Medicine*, 344:539-548.
- X Hua, X Yan, S Yanchopoulos, Y Yang, W Li (2011), “STRAT-VOL: stratified volcano plot for microarray expression analysis”, in preparation.
- JPA Ioannidis (2005), “Microarrays and molecular research: noise discovery?”, *Lancet*, 365:454-455.

- JPA Ioannidis, DB Allison, CA Ball, I Coulibaly, X Cui, AC Culhane, M Falchi, C Furlanello, L Game, G Jurman, J Mangion, T Mehta, M Nitzberg, GP Page, E Petretto, V van Noort (2009), “Repeatability of published microarray gene expression analyses”,
- RA Irizarry, D Warren, F Spencer, IF Kim, S Biswal, BC Frank, E Gabrielson, JGN Garcia, J Geoghegan, G Germino, C Griffin, SC Hilmer, E Hoffman, AE Jedlicka, E Kawasaki, F Martínez-Murillo, L Morsberger, H Lee, D Petersen, J Quackenbush, A Scott, M Wilson, Y Yang, SQ Ye, W Yu (2005), “Multiple-laboratory comparison of microarray platforms”, *Nature Methods*, 2:345-350.
- W Jin, RM Riley, RD Wolfinger, KP White, G Passador-Gurgel, G Gibson (2001), “The contributions of sex, genotype and age to transcriptional variance in *Drosophila melanogaster*”, *Nature Genetics*, 29:389-395.
- C Kim, S Paik (2010), “Gene-expression-based prognostic assays for breast cancer”, *Nature Reviews Clinical Oncology*, 7:340-347.
- RR Kitchen, VS Sabine, AH Sims, EJ Macaskill, L Renshaw, JS Thomas, JI van Hemert, JM Dixon, JMS Bartlett (2010), “Correcting for intra-experiment variation in Illumina BeadChip data is necessary to generate robust gene-expression profiles”, *BMC Genomics*, 11:134.
- WP Kuo, F Liu, J Trimarchi, C Punzo, M Lombardi, J Sarang, ME Whipple, M Maysuria, K Serikawa, SY Lee, D McCrann, J Kang, JR Shearstone, J Burke, DJ Park, X Wang, TL Rector, P Ricciardi-Castagnoli, S Perrin, S Choi, R Bumgarner, JH Kim, GF Short, III, MW Freeman, B Seed, R Jensen, GM Church, E Hovig, CL Cepko, P Park, L Ohno-Machado, TK Jenssen (2006), “A sequence-oriented comparison of gene expression measurements across different hybridization-based technologies”, *Nature Biotechnology*, 24:832-840.
- JE Larkin, BC Frank, H Gavras, R Sultana, J Quackenbush (2005), “Independence and reproducibility across microarray platforms”, *Nature Methods*, 2:337-344.
- JT Leek, RB Scharpf, HC Bravo, D Simcha, B Langmead, WE Johnson, D Geman, K Baggerly, RA Irizarry (2010), “Tackling the widespread and critical impact of batch effects in high-throughput data”, *Nature Reviews Genetics*, 11:733-739.

- W Li (2006), “The-more-the-better and the-less-the-better”, *Bioinformatics*, 22:2187-2188.
- W Li ((2008), “Three lectures on case-control genetic association analysis”, *Briefings in Bioinformatics*, 9:1-13.
- W Li and Y Yang (2002), “How many genes are needed for a discriminant microarray data analysis” in *Methods of Microarray Data Analysis* eds. SM Lin and KF Johnson (Kluwer Academic), pp.137-150.
- JG Liao and KV Chin (2007), “Logistic regression for disease classification using microarray data: model selection in a large p and small n case”, *Bioinformatics*, 23:1945-1951.
- K Miclaus, M Chierici, C Lambert, L Zhang, S Vega, H Hong, S Yin, C Furlanello, R Wolfinger, F Goodsaid (2010), “Variability in GWAS analysis: the impact of genotype calling algorithm inconsistencies”, *The Pharmacogenomics Journal*, 10:324-335.
- JS Morey, JC Ryan, FM van Dolah (2006), “Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR”, *Biological Procedures Online*, 8:175-193.
- W Pan (2002), “A comparative review of statistical methods for discovering differentially expressed genes in replicated microarray experiments”, *Bioinformatics*, 18:546-554.
- PJ Park, YA Cao, SY Lee, JW Kim, MS Chang, R Hart, S Choi (2004), “Current issues for DNA microarrays: platform comparison, double linear amplification, and universal RNA reference”, *Journal of Biotechnology*, 112:225-245.
- TA Patterson, EK Lobenhofer, SB Fulmer-Smentek, PJ Collins, TM Chu, W Bao, H Fang, ES Kawasaki, J Hager, IR Tikhonova, SJ Walker, L Zhang, P Hurban, F de Longueville, JC Fuscoe, W Tong, L Shi, RD Wolfinger (2006), “Performance comparison of one-color and two-color platforms within the Microarray Quality Control (MAQC) project”, *Nature Biotechnology*, 24:1140-1150.
- Y Pawitan, S Michiels, S Koscielny, A Gusnanto, A Ploner (2005), “False discovery rate, sensitivity and sample size for microarray studies”, *Bioinformatics*, 21:3017-3024.

- SL Pomeroy, P Tamayo, M Gaasenbeek, LM Sturla, M Angelo, ME McLaughlin, JYH Kim, LC Goumnerova, PM Black, C Lau, JC Allen, D Zagzag, JM Olson, T Curran, C Wetmore, JA Biegel, T Poggio, S Mukherjee, R Rifkin, A Califano, G Stolovitzky, DN Louis, JP Mesirov, ES Lander, TR Golub, (2002), "Prediction of central nervous system embryonal tumour outcome based on gene expression", *Nature*, 415:436-442.
- J Quackenbush (2002), "Microarray data normalization and transformation", *Nature Genetics*, 32:496-501.
- A Reiner, D Yekutieli, Y Benjamini (2003), "Identifying differentially expressed genes using false discovery rate controlling procedures", *Bioinformatics*, 19:368-375.
- MD Robinson, DJ McCarthy, GK Smyth (2010), "edgeR: a Bioconductor package for differential expression analysis of digital gene expression data", *Bioinformatics*, 26:139-140.
- EE Schadt, MD Linderman, J Sorenson, L Lee, GP Nolan (2010), "Computational solutions to large-scale data management and analysis", *Nature Reviews Genetics*, 11:647-657.
- M Schena, RA Heller, TP Theriault, K Konrad, E Lachenmerier, RW Davis (1998), "Microarrays: biotechnology's discovery platform for functional genomics", *Trends in Biotechnology*, 16:301-306.
- M Sirota, MA Schaub, S Batzoglou, WH Robinson, AJ Butte (2009), "Autoimmune disease classification by inverse association with SNP alleles", *PLoS Genetics*, 5:e1000792.
- GW Snedecor and WG Cochran (1989), *Statistical Methods*, eighth edition (Iowa State University Press: Ames, IW).
- V Sharov, KY Kwong, B Frank, E Chen, J Hasseman, R Gaspard, Y Yu, I Yang, J Quackenbush (2004), "The limits of log-ratios", *BMC Biotechnology*, 4:3.
- L Shi, W Tong, F Goodsaid, FW Frueh, H Fang, T Han, JC Fuscoe, DA Casciano (2004), "QA/QC: challenges and pitfalls facing the microarray community and regulatory agencies", *Expert Review of Molecular Diagnostics*, 4:761-777.

- L Shi, W Tong, H Fang, U Scherf, J Han, RK Puri, FW Frueh, FM Goodsaid, L Guo, Z Su, T Han, JC Fuscoe, ZA Xu, TA Patterson, H Hong, Q Xie, RG Perkins, JJ Chen, DA Casciano (2005), "Cross-platform comparability of microarray technology: Intra-platform consistency and appropriate data analysis procedures are essential", *BMC Bioinformatics*, 6(suppl 2):S12.
- L Shi, LH Reid, WD Jones, R Shippy, J AWarrington, SC Baker, PJ Collins, F de Longueville, ES Kawasaki, KY Lee, Y Luo, YA Sun, JC Willey, RA Setterquist, GM Fischer, W Tong, YP Dragan, DJ Dix, FW Frueh, FM Goodsaid, D Herman, RV Jensen, CD Johnson, EK Lobenhofer, RK Puri, U Scherf, J Thierry-Mieg, C Wang, M Wilson, PK Wolber, L Zhang, W Slikker, Jr, MAQC Consortium (2006), "The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements", *Nature Biotechnology*, 24:1151-1161.
- DK Slonim (2002), "From patterns to pathways: gene expression data analysis comes of age", *Nature Genetics*, 32(suppl):502-508.
- P Stafford (2008), ed. *Methods in Microarray Normalization* (CRC Press).
- C Steinhoff, M Vingron (2006), "Normalization and quantification of differential expression in gene expression microarrays", *Briefings in Bioinformatics*, 7:166-177.
- JD Storey (2003), "The positive false discovery rate: a Bayesian interpretation and the q -value", *Annals of Statistics*, 31:2013-2035.
- JD Storey, R Tibshirani (2003), "Statistical significance for genomewide studies", *Proceedings of National Academy of Sciences*, 100:9440-9445.
- RB Stoughton (2005), "Application of DNA microarrays in biology", *Annual Review of Biochemistry*, 74:53-82.
- V Trevino, F Falciani, HA Barrera-Saldana (2007), "DNA Microarrays: a powerful genomic tool for biomedical and clinical research", *Molecular Medicine*, 13:527-541.
- Y Tu, G Stolovitzky, U Klein (2002), "Quantitative noise analysis for gene expression microarray experiments", *Proceedings of National Academy of Sciences*, 99:14031-14036.

- VG Tusher, R Tibshirani, G Chu (2001), "Significance analysis of microarrays applied to the ionizing radiation response", *Proceedings of National Academy of Sciences*, 98:5116-5121.
- J Vandesompele, K de Preter, F Pattyn, B Poppe, N van Roy, A de Paepe, F Speleman (2002), "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes", *Genome Biology*, 3:34.
- MJ van de Vijver, YD He, LJ van't Veer, H Dai, AAM Hart, DW Voskuil, GJ Schreiber, JL Peterse, C Roberts, MJ Marton, M Parrish, D Atsma, A Witteveen, A Glas, L Delahaye, T van der Velde, H Bartelink, S Rodenhuis, ET Rutgers, SH Friend, R Bernards (2002), "A gene-expression signature as a predictor of survival in breast cancer", *New England Journal of Medicine*, 347:1999-2009.
- BL Welsh (1947), "The generalization of 'Student's' problem when several different population variances are involved", *Biometrika*, 34:28-35.
- DM Witten, R Tibshirani (2007), "A comparison of fold-change and the t-statistic for microarray data analysis", Department of Statistics, Stanford University technical report.
- EP Xing, MI Jordan, RM Karp (2001), "Feature selection for high-dimensional genomic microarray data", in *Proceedings of the Eighteenth International Conference of Machine Learning (ICML 2001)*, eds. CE Brodley, AP Danyluk (Morgan Kauffmann), pp. 601-608.
- YH Yang, T Speed (2002), "Design issues for cDNA microarray experiments", *Nature Reviews Genetics*, 3:579-588.
- TJ Yeatman (2009), "Predictive biomarkers: identification and verification", *Journal of Clinical Oncology*, 27:2743-2744.
- RA Young (2000), "Biomedical discovery with DNA arrays", *Cell*, 102:9-15.
- S Zhang, J Cao (2009), "A close examination of double filtering with fold change and t test in microarray analysis", *BMC Bioinformatics*, 10:402.
- C Zhao, ML Bittner, RS Chapkin, ER Dougherty (2010), "Characterization of the effectiveness of reporting lists of small feature sets relative to the accuracy of the prior biological knowledge", *Cancer Informatics*, 9:49-60.